Role of the K-Antigen Subgroup of Capsular Polysaccharides in the Early Recognition Process Between *Rhizobium meliloti* and Alfalfa Leaves

Isabelle Becquart-de Kozak,¹ Bradley L. Reuhs,² Dominique Buffard,^{1,3} Colette Breda,¹ John S. Kim,² Robert Esnault,^{1,3} and Adam Kondorosi¹.

¹Institut des Sciences Végétales, Centre National de la Recherche Scientifique, Avenue de la Terrasse, F-91198 Gif-sur-Yvette Cedex, France; ²Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Road, Athens, Georgia 30602 U.S.A.; ³Université Paris 7, F-75005 Paris, France

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We used a model system to investigate the induction of plant genes by bacterial surface compounds. We have infiltrated alfalfa leaves with wild-type *Rhizobium meliloti* strain Rm41 and mutant derivatives, which are deficient in the production of exopolysaccharides (EPS), capsular K-polysaccharides (KPS or K-Antigens), or both. We have shown that wild-type *R. meliloti* was able to induce transcript accumulation of genes encoding enzymes of the isoflavonoid biosynthetic pathway: chalcone synthase (CHS), chalcone reductase (CHR), and isoflavone reductase (IFR). Kinetics of these transcript accumulations were biphasic, occurring 0.75 to 1.5 h and 6 to 30 h after treatment. The *exoB* derivative of Rm41 (strain AK631), which is unable to produce either EPS I or EPS II, was still able to induce very rapid (45-min posttreatment) transcript accumulation of CHS and CHR genes. These results suggested that EPS were not involved in this induction. We then tested three *R. meliloti* fix-23 mutants (strains PP699, PP711, and PP671), which were deficient in KPS production as shown by PAGE and immunoblot analyses. In contrast to the results obtained with the mutant strains, infiltration of alfalfa leaves with purified KPS, isolated from strain AK631, led to a very rapid induction of the CHS and CHR genes. Therefore, we hypothesize that KPS may play a role in the early recognition of rhizobia by the leaf cells.

Additional keywords: bacterial surface polysaccharides, gene expression, *Medicago sativa*.

The interaction between *Rhizobium meliloti* and alfalfa (*Medicago sativa*) results in the formation of an indeterminate root nodule, in which the bacteria differentiate into nitrogen-fixing bacteroids. Nod factors, a family of sulfated and acylated glucosamine oligosaccharides, have been identified as the rhizobial signals promoting nodule induction (Lerouge et al. 1990; Roche et al. 1991; Schultze et al. 1992). These molecules are able to elicit various responses in the host plant roots, such as deformation of root hairs, cortical cell proliferation, and differentiation into nodule structures (Lerouge et al. 1990; Schultze et al. 1992; Truchet et al. 1991). Nod factor production is induced by specific plant-derived flavonoids

(reviewed by Göttfert 1993). This signal exchange constitutes one of the early recognition steps in the plant-microbe interaction.

Other molecules of the *Rhizobium* partner that are considered as possible signals in symbiosis include the bacterial cell wall components. The cell wall of gram-negative bacteria consists of the cytoplasmic membrane, or inner membrane, and the outer membrane, as well as the periplasmic space (reviewed by Whitfield and Valvano 1993). The outer membrane is firmly attached via lipoproteins to the peptidoglycan layer, and consists of an asymmetric lipid bilayer with the outer leaflet being formed by the lipid part of lipopolysaccharide (LPS) and the inner leaflet being composed of phospholipids and lipoproteins. LPS molecules comprise three major moieties: the lipid A membrane anchor, a core oligosaccharide, which is linked to the lipid A via 3-deoxy-D-manno-2-octulosonic acid (Kdo), and an antigenic polysaccharide (O antigen). The bacterial cell surface is often covered with an extracellular layer of polysaccharides (CPS), which is commonly termed the capsule (Boulnois and Jann 1989). One subgroup of these CPS (K-Antigens, K-polysaccharides, or KPS) in *Escherichia coli* has been classified according to the presence of Kdo as the acidic component, among other criteria (Jann and Jann 1990). Similar polysaccharides have been identified in *R. fredii* and *R. meliloti* (Reuhs et al. 1994). The extracellular matrix of the bacteria consists of acidic (negatively charged) exopolysaccharides (EPS), which is released into the cell's milieu as extracellular slime.

The different polysaccharides may play important roles in interactions between the bacterium and its environment, including eukaryotic hosts: LPS and CPS have been proposed to protect bacterial pathogens against animal host defenses (Lüderitz et al. 1982; Shafer et al. 1984; Jann and Jann 1990). The LPS may also protect pathogenic bacteria against plant defenses. Mutants of the plant pathogen *Erwinia chrysanthemi* having severe LPS alterations exhibit avirulence which may result from bacterial sensitivity to plant defenses (Schoonejans et al. 1987). Strains of *Pseudomonas solanacearum*, another plant pathogen, are avirulent when both LPS and EPS are altered (Hendrick and Sequeira 1984). Severe disease symptoms, such as water-soaking and wilting, caused by phytopathogenic bacteria are often correlated with ability of pathogens to produce EPS. These molecules can also promote coloniza-

Corresponding author: R. Esnault; E-mail: Esnault@isv.cnrs-gif.fr

tion and enhance survival of some bacteria within host tissues (for review see Denny 1995). CPS are known to play a variety of roles in the interaction between bacteria and their environment. They are particularly important for animal pathogens that encounter the mammalian immune system. Some CPS may resemble host molecules and as such are poorly immunogenic, allowing the bacteria to avoid the host's specific immune system. In that case, the capsule has a protective function: It can play a role in bacterial evasion of host defenses in having a camouflage effect (Jann and Jann 1990).

In *Rhizobium*-legume symbiosis, bacterial surface polysaccharides are required for infection thread formation and release of bacteria into infected plant cells (reviewed by Noel 1992). Genetic studies have shown that LPS molecules were required by *R. leguminosarum* bv. *phaseoli* for normal nodulation and nitrogen fixation (Diebold and Noel 1989). Mutants of *R. meliloti*, *R. leguminosarum* bv. *trifolii*, and *R. leguminosarum* bv. *viciae* that produced very little or none of the O-antigen were also abnormal in nodule development, indicating that the production of LPS O-antigen is required for indeterminate nodulation (Brink et al. 1990; de Maagd et al. 1989; Clover et al. 1989). Dazzo et al. (1991) have shown that LPS from *R. leguminosarum* bv. *trifolii* triggered metabolic events in white clover root hairs, such as concentration-dependent stimulation of infection thread development and changes in cytoplasmic streaming and protein composition. They concluded that these LPS-root hair interactions contribute to the complex signalling process which occurs during successful infection, leading to development of nitrogen-fixing *Rhizobium*-legume symbiosis (Dazzo et al. 1991). The slow migrating form of LPS (LPS I) has been thought to be a signal for the plant, or to physically mask other cell surface components, thereby protecting the bacterium from plant defense mechanism. LPS I⁻ VF39 mutants of *R. leguminosarum* bv. *trifolii*, presenting only few LPS molecules of low molecular weight, induced white infected nodules, but without nitrogen-fixing activity (Priefer 1989).

Djordjevic et al. (1987) have shown that exogenous addition of EPS or specific EPS-derived oligosaccharides to *Leucaena leucocephala* var. Peru, *Macroptilium atropurpureum* or *Trifolium repens* (L.) cv. 5826, together with *Exo*⁻ mutants, resulted in the correction of the inability of these mutants to induce nitrogen-fixing nodules, which suggested that EPS played an active and direct role in the nodulation process. The primary acidic exopolysaccharide (succinoglycan or EPS I) of *R. meliloti* has also been proposed to be a recognition signal (Urzainqui and Walker 1992). EPS I⁻ mutants of strain Rm1021, form Fix⁻ nodules, devoid of bacteroids, because they are unable to elicit the shepherd's crook or infection thread (Leigh et al. 1985). A second exopolysaccharide, EPS II or galactoglucan, is produced in *R. meliloti* strains containing a mutation in the chromosomally located genes *expR* (Glazebrook and Walker 1989) or *mucR* (Zhan et al. 1989). These genes have been assumed to be negative regulators of both, EPS I and EPS II synthesis (Leigh et al. 1993; Gonzalez et al. 1993). *R. meliloti* strains that produce a specific size range of EPS II, induce and invade nodules, and fix nitrogen, suggesting that EPS II can perform the roles in nodule development which are normally performed by EPS I (Her et al. 1990; Gonzalez et al. 1996). In addition, purified EPS II promoted the infection of alfalfa by EPS⁻ mutants at very low

levels, indicating an active signal role for this product (Gonzalez et al. 1996).

In contrast to Rm1021, Rm41 EPS⁻ mutants (i.e., AK631) remain Fix⁺ on alfalfa (Putnoky et al. 1988). However mutations in the *fix-23* region of AK631 result in Fix⁻ empty nodules, suggesting that another component may promote infection by strain AK631. Upon analysis, the chromatographic pattern of Kdo containing polysaccharides from the mutant strains was found to be altered, leading to the conclusion that LPS may substitute for the EPS in this system (Putnoky et al. 1990; Williams et al. 1990). Part of the *fix-23* region has been sequenced, and the predicted protein products from six open reading frames (ORFs) exhibit strong homology and similar organization to those of the rat fatty acid synthase multifunctional enzyme domains (Petrovics et al. 1993). Phage inactivation studies and analysis of partially purified cell extracts have shown that the proteins encoded by this region are involved in the synthesis of a capsular polysaccharide, which is distinct from classic LPS and from secreted EPS; apparently it is this polysaccharide that substitutes the EPS in the *R. meliloti*-alfalfa system (Petrovics et al. 1993; Reuhs et al. 1993; Reuhs et al. 1995). This polysaccharide was shown to be structurally analogous to one subclass of K-antigens produced by *Escherichia coli*, and has been also described in *R. fredii* (Reuhs et al. 1993); such products are now known to be common to *Rhizobia* (Reuhs et al., unpublished).

It has been proposed that surface polysaccharides might act by controlling the elicitation of host defense mechanisms, which have been shown to occur during nodule infection by surface polysaccharide mutants (Noel 1992; Leigh and Coplin 1992). This may then be termed an early recognition step, regardless of what end products the plant may yield. To test this hypothesis, we have infiltrated alfalfa leaves with different strains of *R. meliloti*: wild-type, live, or heat-killed or mutants affected either in EPS production, or in K-polysaccharide production or export, as well as purified KPS isolated from cultured cells. We have analyzed transcript accumulation of genes encoding enzymes, chalcone synthase (CHS), chalcone reductase (CHR), and isoflavone reductase (IFR), involved in the isoflavonoid pathway.

In this paper, we show a rapid accumulation of CHS, and CHR transcripts due to the presence of KPS on the bacterial surface.

RESULTS

To test whether the wild-type *R. meliloti* strain is capable of inducing any defense-related reaction in the infiltrated leaves, we analyzed the expression of the CHS, CHR, and IFR genes involved in the isoflavonoid pathway.

Infiltration of a suspension of the wild-type *R. meliloti*, strain Rm41 into alfalfa leaves did not provoke any macroscopic reaction, such as decoloration of the infected tissue or necrosis. Furthermore, the bacterial population did not increase in the leaves, but remained stable until 8 days after treatment, and 10 days after infiltration no surviving bacteria were detected (data not shown).

Expression of the CHS, CHR, and IFR genes in leaves infected by wild-type *R. meliloti*.

We focused our expression analyses on the very early stages of plant-bacterium recognition and therefore a time course ex-

periment, with an emphasis on the very beginning of the interaction was performed.

Transcript accumulation in leaves infiltrated with *R. meliloti* (Rm41).

Accumulation of transcripts of the three analyzed genes occurred as soon as 45 min after infiltration (Fig. 1A). The striking characteristic of the induction kinetics was that all

Fig. 1. Expression of genes encoding chalcone synthase (CHS), chalcone reductase (CHR), and isoflavone reductase (IFR) in alfalfa leaves after infiltration with: **A**, *Rhizobium meliloti* strain Rm41 and, **B**, heat-killed *R. meliloti* strain Rm41. Densitometric analysis of autoradiograms are presented. To exclude loading variations, induction factors have been calculated (as described in Materials and Methods) by correcting densitometric values obtained from each probe by the appropriate constitutive values.

three genes were expressed with a biphasic kinetics. The first peak occurred from 45 min (the first analyzed time point) to 3 h after treatment with a maximum of transcript accumulation at 1.5 h after injection and the second one was detected between 6 and 30 h postinfiltration.

Even though the three genes seemed to be coordinately activated, their respective transcription levels differed. In the first phase, the CHS transcripts accumulated the most rapidly, as soon as 45 min after infiltration and reached its maximum 1.5 h after treatment, with approximately a 11-fold increase in the accumulated transcript levels. In contrast, the second phase transcript levels were different for the three genes; 2.5-fold, 9-fold, and 13-fold increases were detected 30 h after treatment, for the CHS, CHR and IFR genes, respectively.

Transcript accumulation in leaves infiltrated with heat-killed Rm41.

The above results indicated that, in our experimental conditions, alfalfa leaves responded to infiltration with Rm41 bacteria with a biphasic accumulation of transcripts from genes belonging to the isoflavonoid pathway. We hypothesized that these two phases might result from different types of elicitation. The first one, appearing very rapidly (detectable as soon as 45 min) could result from some preexisting bacterial surface compounds. The second one might be due to compounds released by dying bacteria after infiltration of bacterial sus-

Fig. 2. Expression of the CHS, CHR, and IFR genes upon infiltration of alfalfa leaves with *Rhizobium meliloti* strain AK631. Induction factors were calculated from densitometric values of Northern blots hybridized with the CHS, CHR, and IFR probes (as described in Fig. 1).

Table 1. Bacterial strains used in this study

Strain	Genotype	Symbiotic phenotype	EPS production	External KPS production	Reference
Rm41	exo ⁺ fix-23 ⁺	Fix ⁺	+	+	a, b
AK631	exoB631 fix-23 ⁺	Fix ⁺	—	—	a, b
PP699	exo ⁺ fix-23::Tn5(699)	Fix ⁺	+	—	a,
PP711	exo ⁺ fix-23::Tn5(711)	Fix ⁺	+	—	a, This work
PP671	exoB631 fix-23::Tn5(671)	Fix [—]	—	—	a, b, This work

^a Putnoky et al. 1990.

^b Petrovics et al. 1993; Reuhs et al. 1993.

pensions or to newly synthesized compounds produced by living bacteria which might act directly or indirectly as elicitors. To test this hypothesis we infiltrated an autoclaved (i.e., heat-killed) Rm41 suspension into the leaves.

Upon infiltration with heat-killed cells, the accumulation of CHS transcripts was more transient in the first phase of gene induction, peaking 45 min after infiltration at a significantly higher level (12-fold increase, Fig. 1B) when compared to the treatment with live Rm41 (7-fold increase, Fig. 1A) at the same time point. As with live cells, CHR and IFR transcript accumulations peaked 1.5 h after treatment.

The major difference from results obtained after infiltration with live Rm41 was the lack of the second phase of gene induction: no significant (i.e., <3-fold increase) transcript accumulation was detectable from 4.5 h after treatment. These results are in line with our hypothesis that the first phase of transcript accumulations might be the result of recognition of some surface compounds.

EPS is not involved in rapid accumulation of transcripts of CHS and CHR genes.

To test the possible implication of EPS in the induction of transcript accumulation of genes of the isoflavonoid biosynthetic pathway, we infiltrated alfalfa leaves with an *exoB*⁻ derivative of *R. meliloti*, Rm41 (AK631), which was shown to induce Fix⁺ nodules on alfalfa roots (Putnoky et al. 1988). As the product of the *exoB* gene is a UDP-glucose-4'-epimerase (Canter-Cremers et al. 1990), AK631 is unable to produce either EPS I or EPS II, both of which contain galactose (Putnoky et al. 1990).

Transcript accumulations of CHS and CHR genes were observed after leaf infiltration of the nonmucoid strain (AK631), whereas significant accumulation (>3-fold increase) of IFR transcripts was not detected (Fig. 2). CHS transcripts accumulated at high levels at 45 min after treatment, with a 32-fold increase as compared to the control treatment, and then decreased rapidly. This level of CHS transcript accumulation was higher than after Rm41 treatment, which lead only to a 7-fold increase (Fig. 1A). Forty-five minutes posttreatment, CHR transcripts accumulated to a lesser extent: a 9-fold increase was detected and their maximum of accumulation was reached 3 h after treatment with a 17-fold increase. Although the levels of transcript were higher, the kinetics of induction were similar to those from heat-killed Rm41 cells.

These results indicate that the EPS-defective strain (AK631) retained the ability for inducing transcript accumulation of CHS and CHR genes, suggesting that neither EPS I nor EPS II were implicated in the very rapid accumulation of these transcripts. As no induction of IFR resulted from the leaf infiltration of AK631, EPS may be involved in this response.

Involvement of KPS in accumulation of transcripts of CHS, CHR, and IFR genes.

KPS production of R. meliloti mutants affected in the fix-23 region.

To analyze the possible involvement of KPS in the very rapid accumulation of transcripts encoding genes of the flavonoid pathway, we tested *R. meliloti* strains mutated in the *fix-23* region. It has been previously shown that strains of *R. meliloti* mutated in the first six ORFs of *fix-23* did not express

high molecular weight K-polysaccharides (Petrovics et al. 1993). Two of them, PP666 and PP674 (derivatives of AK631), carry adjacent Tn5 insertions in the same open reading frame (ORF1) of the first complementation unit (Putnoky et al. 1990; Petrovics et al. 1993). In the present work, we tested three *R. meliloti* mutants of the *fix-23* region, described in Table 1: PP699, which has an EPS⁺ background, and in which the Tn5 insertion has the same localization as the PP666 mutant. The other two mutants were PP711 (EPS⁺ background) and PP671 (EPS⁻ background), carrying Tn5 insertions in the second and fourth complementation units of the *fix-23* region, respectively (Putnoky et al. 1990). Prior to the plant assays, we performed an analysis of the KPS production by these mutants.

The results of PAGE analysis of hot phenol-water extracts from the wild-type and mutant strains are presented in Figure 3. The uppermost arrow indicates the position of an unidentified component, which is present in all strains, but is most apparent in the mutants. The low-mobility smear (region I), which results from the high molecular weight K-polysaccharides, is only detectable when the gel is prestained with the Alcian blue, a cationic dye. This component was clearly reduced in each *fix-23* mutant tested (Fig. 3, lanes 2, 4, 5). Region II (also Alcian blue specific), consisting of a ladder

Fig. 3. PAGE analysis of hot phenol-water extracted material from AK631 (well 1), PP671 (well 2), Rm41 (well 3), PP699 (well 4), and PP711 (well 5). The gel was stained using the Alcian blue silver-stained procedure (Reuhs et al. 1993). The arrow (a) shows the location of a yet unidentified component. Very high molecular weight K-polysaccharides (KPS) of strain PP711 have probably comigrated with this unidentified material (lane 5). Region I shows the location of high molecular weight KPS. The group of three arrows (b, c, and d) shows the location of smooth LPS forms. Region II shows the location of intracellular low molecular weight KPS. The arrow (e) shows the location of rough LPS, and arrow (f), the location of *exoB*⁻ specific rough LPS.

pattern, results from a lower molecular weight form (i.e., subunits) of the K-polysaccharides, which is harbored intracellularly. AK631, in contrast to Rm41, yields less of these products upon extraction, suggesting that the lack of EPS production by AK631 results in a more rapid incorporation of the subunits into exportable polysaccharides. These data are in agreement with those reported for PP666 and PP674 (Petrovics et al. 1993). Due to the *exxB* mutation, clear differences were seen in LPS patterns between the AK631 group (Fig. 3, lanes 1, 2) and the Rm41 group (Fig. 3, lanes 3, 4, 5) but the respective *fix-23* mutants (Fig. 3, lanes 2, 4, 5) were

unaltered in LPS mobility compared to the parent strains (Fig. 3, lanes 1, 3).

Polyclonal antibodies raised against wild-type strain Rm41 were used to detect the presence of KPS in an immunoblot analysis of hot phenol-water extracts using these antibodies (Fig. 4). As PAGE analyses are based on extraction of total polysaccharides, the antiserum was used in its native form, and also after preabsorption with whole cells, which removes antibodies to components on the cell surfaces.

The first analysis used the Rm41 antiserum in its native form and showed that wild-type K-polysaccharides from

Fig. 4. Immunoblots of hot phenol-water extracted material from AK631 (well 1), PP671 (well 2), Rm41 (well 3), PP699 (well 4), and PP711 (well 5). Arrows and regions I and II represent the same polysaccharides as already described in the legend of Figure 3. Immunoblots were performed using antiserum to *Rhizobium meliloti* strain Rm41 (Panel A), the same antiserum preabsorbed with *R. meliloti* strain AK631 (Panel B), *R. meliloti* strain PP671 (Panel C), *R. meliloti* strain PP711 (Panel D).

AK631 and Rm41 (Fig. 4A, lanes 1, 3) were efficiently detected, whereas the presence of these polysaccharides was not shown for the mutant strains (Fig. 4A, lanes 2, 4, 5). Some very high molecular weight, yet unidentified material cross-reacted with the antibodies in strain PP711 (Fig. 4A, lane 5), and this would indicate that some very high molecular weight KPS comigrated with the unidentified compounds detected in Fig. 3 (arrow a), this has been confirmed in another study (Kiss et al., unpublished). As anti-Rm41 was used in this experiment, there was a stronger response to the LPS from Rm41 mutants than with the AK631 strains, which are deficient in both EPS and LPS production (also shown in the PAGE gel).

Immunoblots were performed with the same polyclonal antiserum after preabsorption with AK631, PP671, and PP711 cells (Fig. 4B, C, and D respectively). No polysaccharides were detected after preabsorption with AK631 (Fig. 4B), indicating that the antibodies directed against common epitopes shared by strains Rm41 and AK631 were eliminated. When

antibodies were preabsorbed with PP671 (Fig. 4C) or PP711 (Fig. 4D) cells, they were still able to react with high molecular weight KPS (region I) in Rm41 (lane 3) and AK631 (lane 1) extracts. These results confirm that mutations in PP671 or PP711 have reduced or eliminated KPS expression on the cell surface. The lower intensity of the immunoreaction observed in Figure 4D, compared to Figure 4C indicates the presence of some high molecular weight KPS on the PP711 cell surface. This was also apparent in Figure 4A. These experiments showed that the three *R. meliloti* strains, mutated in different genes of the *fix-23* region, exhibit only very reduced amounts of K-polysaccharides on their surfaces.

Transcript accumulation after infiltration of R. meliloti mutants affected in KPS production.

Very low levels of transcript accumulation of CHS, CHR, and IFR genes occurred after leaf infiltration with strains PP699 (Fig. 5A), PP711 (Fig. 5B) or PP671 (Fig. 5C). Importantly, there was no rapid transcript accumulation of these genes. A transcript accumulation slightly higher (5-fold increase) than the determined threshold (>3-fold increase), was observed for CHR genes, in PP671-infiltrated leaves, 3 and 4.5 h after treatment. But these transcript accumulations were significantly lower than those obtained after AK631 infiltration (Fig. 2), which lead to 17- and 8-fold increases of CHR genes, 3 and 4.5 h posttreatment, respectively. These results suggested that the K-polysaccharides might be responsible for the observed transcript accumulations after infiltration with wild-type *R. meliloti* (Fig. 1). To test this hypothesis, we employed purified KPS in the same assay system.

Transcript accumulation after infiltration of leaves with KPS.

K-Polysaccharides purified from AK631 were infiltrated at $\approx 8.10^{-6}$ M; this concentration was determined to be an approximation of injected concentrations of KPS present on bacterial surfaces in bacteria infiltration experiments (based

Fig. 5. Expression of CHS, CHR, and IFR genes upon infiltration with *R. meliloti* strains PP699 (A), PP711 (B), and PP671 (C). Induction factors were calculated from densitometric values of Northern blots hybridized with the CHS, CHR, and IFR probes (as described in Fig. 1).

Fig. 6. Expression of the CHS, CHR, and IFR genes upon infiltration of alfalfa leaves with purified K-polysaccharides from *R. meliloti* (AK631). The KPS solution was infiltrated at a concentration of $\approx 8.10^{-6}$ M. Induction factors were calculated from densitometric values of Northern blots hybridized with the CHS, CHR, and IFR probes (as described in Fig. 1).

on LPS/KPS yield per cell from extraction). Accumulation of CHS and CHR mRNAs was observed (Fig. 6), with a very rapid accumulation of CHS transcripts (a 17-fold increase was detected 45 min after treatment). The level of CHS transcripts remained high 1.5 h after infiltration (9-fold increase), but they greatly decreased from 3 h after treatment. CHR mRNAs also accumulated, with 7- and 9-fold increases 1.5 and 3 h, respectively, after injection but the IFR transcripts did not show significant accumulation (>3-fold increase). Furthermore, transcript accumulations of each tested gene followed the same kinetics, whether it resulted from infiltrations of AK631 or KPS purified from this bacterium.

These results indicate that K-polysaccharides are capable of inducing rapid transcript accumulation of the CHS and CHR genes.

DISCUSSION

The aim of this study was to determine whether the bacterial surface components of *R. meliloti* could be involved in the induction of transcript accumulation of alfalfa genes. We have used an alternative approach, other than the root-rhizobia interaction. We have infiltrated alfalfa leaves with various *R. meliloti* strains, either wild-type or mutants that were affected in their surface composition. HPLC analyses revealed that flavonoids detected in control leaves were mainly present in a conjugated form, and that free flavonoids capable of *nod* gene activation were not present, suggesting that Nod factors were probably not involved in these interactions (unpublished).

In this study, we have shown that infiltration of alfalfa leaves with wild-type *R. meliloti* provoked a two-phase kinetics of transcript accumulation of the CHS, CHR, and IFR genes. The first peak was observed 45 min to 1.5 h after treatment and the second one, 6 to 30 h postinoculation. Results obtained after leaf infiltrations with AK631 suspension, showed that this strain was still able to induce very rapid (45 min posttreatment) transcript accumulation of CHS, and CHR genes. These results suggested that, in our experimental model system, neither EPS I nor EPS II was responsible for the early recognition events occurring between bacteria and alfalfa leaves, but that EPS might be necessary for IFR gene induction. Differences observed between levels of gene induction after Rm41 and AK631 infiltrations suggested that the lack of EPS on the bacterial surface has enabled one or several other component(s) to be more accessible and recognized by the plant.

Three mutants of *R. meliloti*, carrying either a single mutation in the *fix-23* region (PP699 and PP711) or two mutations, one in the *fix-23* region and another one in *exoB* (PP671), have been studied. We showed that all of these mutants, carrying mutations in different complementation units of *fix-23* (Petrovics et al. 1993; Kiss et al., unpublished), were affected in their KPS production. Significant levels of external K-polysaccharides were not detected, but small amounts of low molecular weight KPS could still be produced, as it was already reported for other *fix-23* mutants of *R. meliloti* (Petrovics et al. 1993). It is possible that some incompletely polymerized subunits of KPS might still be synthesized in PP711 and PP671 mutants, but without being properly polymerized or exported to the bacterial surface (Kiss et al., unpublished). In our experimental conditions, we detected a small amount of very high molecular weight KPS in one mutant, but

we cannot ascertain that this has a biological significance or was an artefact; however, the presence of this KPS fraction in the PP711 strain did not provoke gene inducing activity. Comparison of the results obtained after infiltrations with Rm41, AK631, KPS deficient mutants or purified KPS suggested that K-polysaccharides were responsible for the very rapid expression of the CHS and CHR genes, observed in the interactions between Rm41 or AK631 and the leaf cells. As the levels of expression of the tested genes were 2-fold lower after KPS infiltration, compared to AK631 inoculation, we can hypothesize that the injected concentration of purified KPS ($\approx 8 \cdot 10^{-6}$ M) did not correspond exactly to the amount of KPS exhibited on the surface of the injected bacteria, or that a specific size-range was required, which was present at lower concentration in the preparation used. Another possibility is that other component(s) of the bacterial surface may act in synergy with KPS and play a fundamental role in the recognition process.

This is the first time that K-polysaccharides were shown to induce expression of genes related to flavonoid production and plant defense. Until now only a few publications appeared on the induction of specific genes, and/or gene products in response to bacterial polysaccharide application to plants, either in the case of pathogenic or symbiotic interactions. Recently, Newman et al. (1995) showed that inoculation of turnip (*Brassica campestris*) leaves with purified LPS from *Xanthomonas campestris* pv. *campestris*, the causal agent of black rot of crucifers, induced accumulation of transcripts for β -1,3-glucanase as soon as 4 h after inoculation and up to 24 h. In the case of symbiotic interactions, Miller et al. (1994) showed that in the soybean cotyledon bioassay, a class of rhizobial oligosaccharides, the cyclic β -1,6-1,3-glucans of *Bradyrhizobium japonicum* USDA 110 were biologically active elicitors of glyceollin and daidzein productions.

The obtained results allowed us to conclude that KPS was linked to the very rapid (45 min to 1.5 h) and transient first phase of CHS and CHR transcript accumulations, observed after Rm41 infiltration. The cause of the second phase of transcript accumulations has yet to be determined. We can hypothesize that the late transcript accumulation of genes of the isoflavonoid pathway might be due to a modified KPS production in planta, or to another compound. Newman et al. (1994), showed that substantial growth of *Xanthomonas* bacteria could release sufficient LPS to trigger β -glucanase transcript accumulation, whereas heat-killed or antibiotic-killed bacteria that failed to grow in planta were inactive. They suggested that release of LPS may occur as a normal process during the growth of phytopathogenic bacteria, as it was suggested for symbiotic bacteria (Dazzo et al. 1991). In planta modifications of cell wall polysaccharides were already reported in the case of extracellular polysaccharides produced by some phytopathogenic bacteria (Denny 1995). In the case of symbiotic bacteria, Reuhs et al. (1994) reported structural modifications in the LPS and KPS of *R. fredii*, in response to host-derived compounds. In addition, Noel et al. (1996) showed that an antigenic change in the LPS of *Rhizobium etli* CFN42 was induced by seed and root exudates of *Phaseolus vulgaris*, and occurred during growth of the bacteria and de novo synthesis of the LPS (Noel et al. 1996). Olsen et al. (1992) also reported that anti-*R. meliloti* monoclonal antibodies (Mabs 8 and 9) which recognize KPS (Reuhs, unpublished results), reacted with free-living cells, but not with bacteroids,

suggesting some in planta modifications of external KPS.

Based on the presented results, we conclude that bacterial KPS may have a significant role in the early recognition of *R. meliloti* cells by alfalfa leaf cells. As IFR genes are not induced by these K-polysaccharides, we cannot conclude that plant defenses have been triggered. Analyses of both enzyme activity and flavonoid production would be very useful. To test whether plant defense responses may occur in the roots, analysis of the early events of recognition in the alfalfa root system is now in progress.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Rhizobium meliloti: strains Rm41 and AK631 (*exoB* derivative of strain Rm41, Putnoky et al. 1990) were grown in TA medium: bactotryptone (Difco, Detroit, MI): 10g l⁻¹, NaCl: 5 g liter⁻¹, yeast extract (Difco): 1 g liter⁻¹; supplemented after sterilization with 1 ml liter⁻¹ 1 M CaCl₂ and 1 ml liter⁻¹ 1M MgSO₄, pH 7.2. The *fix-23* mutant strains of *R. meliloti* (Table 1: PP699, PP711, and PP671, Putnoky et al. 1990) were also grown in TA medium, supplemented with 0.2 g liter⁻¹ of kanamycin.

Bacterial cultures grown at 30°C, and were collected in their exponential phase of growth. After centrifugation for 15 min at 700 g (at 4°C), bacterial pellets were resuspended in 10 mM MgCl₂ at a 10⁹ bacteria per milliliter concentration for infiltration of alfalfa leaves.

Extraction of bacterial polysaccharides and purification of K-polysaccharides.

The extraction and initial purification protocols of bacterial polysaccharides have been previously described (Reuhs et al. 1993). Briefly, the cells were pelleted by centrifugation, washed, repelleted, and extracted with the hot phenol-water method (Carlson and Yadav 1985; Johnson and Perry 1976). The water phase was dialyzed against distilled H₂O and lyophilized. Part of the lyophilized materials was dissolved in sample buffer and used for PAGE analyses and immunoblots.

For purification of KPS, the polysaccharides of crude AK631 cell extract were separated by repeated chromatography on a Sephadex G-150 (Pharmacia, Uppsala, Sweden) column, in 0.2 M NaCl, 1 mM EDTA, 10 mM Tris-base, 0.25% deoxycholic acid buffer, pH 9.25. The eluted fractions were chemically assayed for the presence of Kdo by the thiobarbituric acid (TBA) assay (York et al. 1985), and for hexose with phenol-sulfuric acid (York et al. 1985) and then assayed for LPS by PAGE.

The extracted KPS of AK631 cells were injected at a concentration of $\approx 8 \cdot 10^{-6}$ M (assuming an average size range of 20 to 30 kDa for extracellular KPS). This concentration was determined as an approximation of the injected concentrations of KPS present on bacterial surfaces when bacteria were infiltrated (based on LPS/KPS yield per cell from extraction).

Plant material: growth, bacterial and purified KPS treatments.

Alfalfa (*Medicago sativa* ssp. *sativa* cv. Nagyszénási) plants were grown in clay pots in vermiculite under controlled conditions in a growth chamber (22°C with a 16-h light/8-h dark cycle). Plants were watered twice a day: first with deionized water and second with a nutrient solution (Blondon 1964).

Leaves of 6-week-old plants were infiltrated with an hypodermic syringe, (approximately 50 μ l per leaf), with either bacterial suspensions or purified KPS (from AK631). Control leaves were injected with 10 mM MgCl₂. Leaves were harvested from 45 min to 30 h after treatment and immediately frozen in liquid nitrogen.

RNA isolation and blot analysis.

Total RNA extraction was proceeded according to Hall et al. (1978). Poly (A)⁺ RNA were separated by oligo(dT)-cellulose and 1 μ g per lane was loaded on 6% formaldehyde denaturing electrophoresis gels. Blotting onto Hybond N nylon membrane (Amersham) was performed by capillarity. In order to verify the equal loading of amounts of RNA on the gel, all blots were hybridized with a cDNA probe, obtained from the Kochia plant (*Chenopodiaceae*) and designated as AHS8 (kindly provided by G. Selvaraj, Plant Biotechnology Institute, Saskatoon, Canada). This probe has been shown to hybridize with a constitutively expressed gene. The blots were hybridized with the other probes of interest and after exposition with a Kodak (X-OMAT) film, a computer analysis of the different spots obtained on autoradiograms was performed using the BioImage system from Millipore. For each probe and for each time point, a pre-corrected factor, defined as the ratio between the densitometrical values of the treated samples vs. the control sample, was calculated. Same calculations were realized with the constitutive probe (AHS8) leading to the determination of the "constitutive factors" used for correcting variations between sample loadings. Finally, an induction factor was calculated for each probe and for each time point, by dividing "pre-corrected" factor by the appropriate "constitutive" factor. Induction factors lower than 3 were not considered as significant, according to data obtained in our experimental conditions, from one experiment to another.

PAGE and immunoblotting.

PAGE and immunoblotting protocols have been previously described (Petrovics et al. 1993; Reuhs et al. 1993). PAGE was performed on the phenol-water extracts, using 18% acrylamide gels with deoxycholic acid as a detergent, and gels were Alcian blue/silver stained (Reuhs et al. 1993). For immunochemical analyses, 18% polyacrylamide gels were transferred to Nytran⁺ membrane (Schleicher & Schuell, Keene, NH), using a Trans-Blot SD apparatus (Bio-Rad, Richmond, CA). Blots were incubated with anti-Rm41 or anti-Rm41 preadsorbed with AK631, PP671, or PP711 cells as described in Petrovics et al. (1993), and developed with goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Sigma A-9919) and p-nitrophenyl phosphate as substrate.

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LITERATURE CITED

- Blondon, F. 1964. Contribution à l'étude du développement des graminées fourragères: ray-grass et dactyle. Rev. Gén. Bot. 71:293-381.
Boulnois, G. J., and Jann, K. 1989. Bacterial polysaccharide capsule

- synthesis, export and evolution of structural diversity. *Mol. Microbiol.* 3:1819-1823.
- Brink, B. A., Miller, J., Carlson, R. W., and Noel, K. D. 1990. Expression of *Rhizobium leguminosarum* CFN42 genes for lipopolysaccharide in strains derived from different *R. leguminosarum* soil isolates. *J. Bacteriol.* 172:548-555.
- Canter Cremers, H. C. J., Batley, M., Redmond, J. W., Eydem, L., Breedveld, M., Zevenhuizen, L. P. T. M., Pees, E., Wijffelman, C. A., and Lugtenberg, B. J. J. 1990. *Rhizobium leguminosarum* *exoB* mutants are deficient in the synthesis of UDP-glucose 4'-epimerase. *J. Biol. Chem.* 265:21122-21127.
- Carlson, R. W., and Yadav, M. 1985. Isolation and partial characterization of the extracellular polysaccharides and lipopolysaccharides from the fast-growing *Rhizobium japonicum* USDA205 and its *nod* mutant, HC205, which lacks the symbiotic plasmid. *Appl. Environ. Microbiol.* 50:1219-1224.
- Clover, R. H., Kieber, J., and Signer, E. R. 1989. Lipopolysaccharide mutants of *Rhizobium meliloti* are not defective in symbiosis. *J. Bacteriol.* 171:3961-3967.
- Dazzo, F. B., Truchet, G. L., Hollingsworth, R. I., Hrabak, E. M., Pankratz, H. S., Philip-Hollingsworth, S., Salzwedel, J. L., Chapman, K., Appenzeller, L., Squartini, A., Gerhold, D., and Orgambide, G. 1991. *Rhizobium* lipopolysaccharide modulates infection thread development in white clover root hairs. *J. Bacteriol.* 173:5371-5384.
- de Maagd, R. A., Rao, A. S., Mulders, I. H. M., Goosen-de Roo, L., van Loosdrecht, M. C. M., Wijffelman, C. A., and Lugtenberg, B. J. J. 1989. Isolation and characterization of mutants of *Rhizobium leguminosarum* bv. *viciae* 248 with altered lipopolysaccharides: Possible role of surface charge or hydrophobicity in bacterial release from the infection thread. *J. Bacteriol.* 171:1143-1150.
- Denny, T. P. 1995. Involvement of bacterial polysaccharides in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:173-197.
- Diebold, R., and Noel, K. D. 1989. *Rhizobium leguminosarum* exopolysaccharide mutants: biochemical and genetic analyses and symbiotic behavior on three hosts. *J. Bacteriol.* 171:4821-4830.
- Djordjevic, S. P., Chen, H., Batley, M., Redmond, J. W., and Rolfe, B. G. 1987. Nitrogen fixation ability of exopolysaccharide synthesis mutants of *Rhizobium* sp. strain NGR234 and *Rhizobium trifolii* is restored by the addition of homologous exopolysaccharides. *J. Bacteriol.* 169:53-60.
- Glazebrook, F., and Walker, G. C. 1989. A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. *Cell* 56:661-672.
- Gonzalez, J. E., Glucksmann, M. A., Reuber, T. L., and Walker, G. C. 1993. Pages 203-206 in: Exopolysaccharides and *Rhizobium meliloti*-alfalfa interactions. New horizons in nitrogen fixation. R. Palacios, J. Moira, and W. Newton, eds. Dordrecht, The Netherlands, Kluwer Academic Publishers.
- Gonzalez, J. E., Reuhs, B. L., and Walker, G. C. 1996. Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. *Proc. Natl. Acad. Sci. USA* 93:8636-8641.
- Göttfert, M. 1993. Regulation and function of rhizobial nodulation genes. *FEMS Microbiol. Rev.* 104:39-64.
- Hall, T. C., Ma, Y., Buchbinder, B. U., Pyne, J. W., Sun, S. M., and Bliss, F. A. 1978. Messenger RNA from G1 protein of French bean seeds. *Proc. Natl. Acad. Sci. USA* 75:3196-3200.
- Hendrick, C. A., and Sequeira, L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. *Appl. Environ. Microbiol.* 48:94-101.
- Her, G. R., Glazebrook, J., Walker, G. C., and Reinhold, V. N. 1990. Structural studies of a novel exopolysaccharide produced by a mutant of *Rhizobium meliloti* strain Rm1021. *Carbohydr. Res.* 198:305-312.
- Jann, B., and Jann, K. 1990. Structure and biosynthesis of the capsular antigens of *Escherichia coli*. *Curr. Top. Microbiol. Immunol.* 150:9-42.
- Johnson, D. G., and Perry, M. B. 1976. Improved techniques for the preparation of bacterial lipopolysaccharides. *Can. J. Microbiol.* 22:29-34.
- Leigh, J. A., Battisti, L., Lee, C. C., Ozga, D. A., Zhan, H., and Astete, S. 1993. Role in nodulation and genetic regulation of exopolysaccharide synthesis in *Rhizobium meliloti*. Pages 175-181 in: *Advances in Molecular Genetics of Plant-Microbe Interactions*. E. W. Nester and D. P. S. Verma, eds. The Netherlands, Kluwer Academic Publishers.
- Leigh, J. A., and Coplin, D. L. 1992. Exopolysaccharides in plant-bacterial interactions. *Annu. Rev. Microbiol.* 46:307-346.
- Leigh, J. A., Signer, E. R., and Walker, G. C. 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proc. Natl. Acad. Sci. USA* 82: 6231-6235.
- Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Dénarié, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* 344:781-784.
- Lüderitz, O., Freudenberg, M. A., Galanos, C., Lehmann, V., Rietschel, E. T., and Shaw, D. H. 1982. Lipopolysaccharides of gram-negative bacteria. *Curr. Top. Membr. Trans.* 17:79-151.
- Miller, K. J., Hadley, J. A., and Gustine, D. L. 1994. Cyclic β -1,6-1,3-glucans of *Bradyrhizobium japonicum* USDA 110 elicit isoflavonoid production in the soybean (*Glycine max*) host. *Plant Physiol.* 104:917-923.
- Newman, M. A., Conrads-Strauch, J., Scofield, G., Daniels, M. J., and Dow, J. M. 1994. Defense-related gene induction in *Brassica campestris* in response to defined mutants of *Xanthomonas campestris* with altered pathogenicity. *Mol. Plant-Microbe Interact.* 7:553-563.
- Newman, M. A., Daniels, M. J., and Dow, J. M. 1995. Lipopolysaccharide from *Xanthomonas campestris* induces defense-related gene expression in *Brassica campestris*. *Mol. Plant-Microbe Interact.* 8:778-780.
- Noel, K. D. 1992. Rhizobial polysaccharides required in symbioses with legumes. Pages 341-357 in: *Molecular Signals in Plant-Microbe Communications*. D. P. S. Verma, ed. CRC Press, Boca Raton, FL.
- Noel, K. D., Duelli, D. M., Tao, H., and Brewin, N. J. 1996. Antigenic change in the lipopolysaccharide of *Rhizobium etli* CFN41 induced by exudates of *Phaseolus vulgaris*. *Mol. Plant-Microbe Interact.* 9:180-186.
- Olsen, P., Collins, M., and Rice, W. 1992. Surface antigens present on vegetative *Rhizobium meliloti* cells may be diminished or absent when cells are in the bacteroid form. *Can. J. Microbiol.* 38:506-509.
- Petrovics, G., Putnoky, P., Reuhs, B., Kim, J., Thorp, T. A., Noel, K. D., Carlson, R. W., and Kondorosi, A. 1993. The presence of a novel type of surface polysaccharide in *Rhizobium meliloti* requires a new fatty acid synthase-like gene cluster involved in symbiotic nodule development. *Mol. Microbiol.* 8:1083-1094.
- Priefer, U. B. 1989. Genes involved in lipopolysaccharide production and symbiosis are clustered on the chromosome of *Rhizobium leguminosarum* biovar *viciae* VF39. *J. Bacteriol.* 171:6161-6168.
- Putnoky, P., Grosskopf, E., Cam Ha, D. T., Kiss, G. B., and Kondorosi, A. 1988. *Rhizobium fix* genes mediate at least two communication steps in symbiotic nodule development. *J. Cell Biol.* 106:597-607.
- Putnoky, P., Petrovics, G., Kereszt, A., Grosskopf, E., CamHa, D., Banfalvi, Z., and Kondorosi, A. 1990. *Rhizobium meliloti* lipopolysaccharide and exopolysaccharide can have the same function in the plant-bacterium interaction. *J. Bacteriol.* 172:5450-5458.
- Reuhs, B. L., Carlson, R. W., and Kim, J. S. 1993. *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-D-manno-2-octulosonic-acid containing polysaccharides that are structurally analogous to group II K antigens (capsular polysaccharides) found in *Escherichia coli*. *J. Bacteriol.* 175:3570-3580.
- Reuhs, B. L., Kim, J. S., Badgett, A., and Carlson, R. W. 1994. Production of cell-associated polysaccharides of *Rhizobium fredii* USDA205 is modulated by apigenin and host root extract. *Mol. Plant-Microbe Interact.* 7:240-247.
- Reuhs, B. L., Williams, M. N. V., Kim, J. S., Carlson, R. W., and Côté, F. 1995. Suppression of the *Fix⁻* phenotype of *Rhizobium meliloti* *exoB* mutants by *lpsZ* is correlated to a modified expression of the K polysaccharide. *J. Bacteriol.* 177:4289-4296.
- Roche, P., Lerouge, P., Ponthus, C., and Promé, J. C. 1991. Structural determination of bacterial nodulation factors involved in the *Rhizobium meliloti*-alfalfa symbiosis. *J. Biol. Chem.* 266:10933-10940.
- Schoonejans, E., Expert, D., and Toussaint, A. 1987. Characterization and virulence properties of *Erwinia chrysanthemi* lipopolysaccharide-defective, ϕ EC2-resistant mutants. *J. Bacteriol.* 169:4011-4017.
- Schultze, M., Quiclet-Sire, B., Kondorosi, E., Virelizier, H., Glushka, J. N., Endre, G., Géro, S. D., and Kondorosi, A. 1992. *Rhizobium meliloti* produces a family of sulfated lipo-oligosaccharides exhibiting different degrees of plant host specificity. *Proc. Natl. Acad. Sci. USA* 89:192-196.
- Shafer, W. M., Joiner, K., Guymon, L. F., Cohen, M. S., and Sparling, P. F. 1984. Serum sensitivity of *Neisseria gonorrhoeae*: The role of

- lipopolysaccharide. J. Infect. Dis. 149:175-183.
- Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J. C., and Dénarié, J. 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351:670-673.
- Urzainqui, A., and Walker, G. C. 1992. Exogenous suppression of the symbiotic deficiencies of *Rhizobium meliloti* *exo* mutants. J. Bacteriol. 174:3403-3406.
- Whitfield, C., and Valvano, M. A. 1993. Biosynthesis and expression of cell-surface polysaccharides in Gram-negative bacteria. Adv. Microb. Physiol. 35:135-246.
- Williams, M. N. V., Hollingsworth, R. I., Klein, S., and Signer, E. R. 1990. The symbiotic defect of *Rhizobium meliloti* exopolysaccharide mutants is suppressed by *lpsZ*⁺, a gene involved in lipopolysaccharide biosynthesis. J. Bacteriol. 172:2622-2632.
- York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., and Albersheim, P. 1985. Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol. 118:3-40.
- Zhan, H., Levery, S. B., Lee, C. C., and Leigh, J. A. 1989. A second exopolysaccharide of *Rhizobium meliloti* strain SU47 that can function in root nodule invasion. Proc. Natl. Acad. Sci. USA 86:3055-3059.